ZOOLOGICAL SCIENCE 10: 337-351 (1993)

Annual Patterns of Testicular Development and Activity in the Chinese Bullfrog (*Rana rugulosa* Wiegmann)

YUNG-HSI KAO[1,2], PAUL S. ALEXANDER[2], VIVIAN V. CHENG YANG[2] and JOHN YUH-LIN YU[1,3]

[1]*Endocrinology Laboratory, Institute of Zoology, Academia Sinica, Nankang, Taipei, Taiwan 115, R.O.C. and* [2]*Department of Biology, Tunghai University, Taichung, Taiwan 407, R.O.C.*

ABSTRACT—Annual patterns of testicular development and of testicular and plasma androgen levels in the Chinese bullfrog, *Rana rugulosa* Wiegmann, are reported. The animals were collected from a frog culture farm from November of 1986 through November of 1987, at monthly intervals or more frequently during the breeding season. Proliferation of primary spermatogonia was observed in November-December. In January-February, the number of primary spermatogonia decreased while secondary spermatogonia increased. Primary and secondary spermatocytes showed little changes until February when their number rose sharply. Although every stage of spermatogenesis was present year around, spermatozoa were massively formed in April-June. Thus, the spermatogenetic cycle of *R. rugulosa*, inhabiting subtropical Taiwan, is of continuous type. Leydig cells began to proliferate in late March and were abundant from April through July. Testicular androgen content showed basal values in January-February and August-December, began to increase in late March, peaked in April, and dropped markedly in May. The pattern of plasma androgen levels was similar to that of testicular androgen content, except peaking in middle April-early May and dropped in late June. The unimodal pattern of seasonal changes in plasma androgen levels parallels Leydig cell proliferation, spermatogenesis, testicular androgen content, and reproductive activities. In addition, the testicular weight was negatively correlated with liver weight, but not with fat body weight during an annual reproductive cycle of *R. rugulosa*.

INTRODUCTION

The patterns of reproductive cycle have been investigated in many species of anurans. The annual spermatogenetic pattern in anurans varies from discontinuous spermatogenesis in the temperate zone to continuous spermatogenesis in tropical species [22]. Earlier studies have shown that ranids, one of the major groups of anurans, display a diverse type of spermatogenesis: such as *Rana temporaria, R. arvalis,* and *R. dalmatina* exhibiting the discontinuous spermatogenesis; *R. tigerina, R. esculenta,* and *R. perezi,* the potencially continuous spermatogenesis; and *R. hexadactyla, R. cyanophlyctis,* and *R. catesbeiana,* the continuous spermatogenesis [6, 22, 43, 44, 58].

The secretion patterns of the pituitary gonadotropins in relation to testicular development and activity during a reproductive cycle of anurans have been relatively less studied [13, 19]. It was demonstrated that the circulating luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were highly correlated, respectively, with the changes in plasma androgen levels and in testicular weight during an annual cycle of male toad, *Bufo japonicus* [13]. On the other hand, seasonal changes in testicular and/or circulating androgen levels in relation to spermatogenetic and/or reproductive activity have been established in several anurans, such as *R. esculenta* [7, 38, 43, 56], *R. perezi* [6], *R. catesbeiana* [19, 58], *B. japonicus* [13], *B. mauritanicus* [48], *Pachymedusa*

Accepted December 3, 1992
Received August 18, 1992
[3] Reprint requests should be sent to: Dr. J.Y.L. Yu, Endocrinology Laboratory, Institute of Zoology, Academia Sinica, Nankang, Taipei, Taiwan 115, R.O.C.

dacnicolor [41], *R. ridibunda* [55], and *Rana occipitalis* [17]. However, the annual patterns of circulating androgen levels in anurans exhibit a great variation of species specificity.

The Indian bullfrog (*R. tigerina* Daudin) was reported to show a potentially continuous spermatogenetic cycle [1, 45]. Correlations of the androgen levels in testis and in blood with spermatogenesis and reproductive activity during an annual cycle of this species were, however, not investigated.

The Chinese bullfrog, *R. rugulosa,* is phylogenically closely related to the Indian bullfrog, *R. tigerina* [9, 20, 39]. Little information has been available in male *R. rugulosa,* inhabiting subtropical Taiwan, and central and southern China, with respect to its patterns of reproductive cycle: spermatogenetic cycle, androgen levels, breeding activity, secondary sexual characters, and changes in various organs in association with reproduction, such as liver and fat body. We have conducted a series of studies investigating the annual reproductive patterns of *R. rugulosa* in both males and females. We report here the annual patterns of male *R. rugulosa* with respect to: 1) the changes in the sizes of body, testis, liver, and fat body; 2) the spermatogenetic cycle, and the changes in the proliferation of Leydig cells; 3) the androgen levels in testis and plasma; and 4) the correlation of plasma and testicular androgen levels with spermatogenesis and other reproductive activity.

MATERIALS AND METHODS

Study site

Rana rugulosa were collected from a frog culture farm located in Tungshih (23°40′N, 120°15′E), Yunlin County, Central Taiwan. Briefly, the frogs were raised in an open pond which was surrounded with fishing nets. They were fed with "frog food" manufactured by Fu-Shou Food Co., Taichung, and the natural preys as well. During hibernation, the frogs were dormant under digged earth caves or the hayricks of rice and woody box.

Chorussing of male frogs was heard from March through July at the frog culture farm, and was occasionally heard in August and September. Young tadpoles with external gills were first observed in late May, and metamorphosing froglets were first observed in early June.

Data on rainfall, temperature, and photoperiod at Tungshih, are summarized in Fig. 1. The period between March and September, 1987 was the rainy season. The lowest monthly mean air temperature was 15.4°C in January and the highest was 28.5°C in August. Monthly minimum air temperature averaged about 5°C in January and February. The photoperiod was shortest in December and longest in June. Shortest and longest days had about three hours difference in photoperiod.

Collection of blood and tissues

Monthly samples of 12–25 sexually mature male frogs (hatched in April, 1986) were collected from November, 1986 through November, 1987, except for sampling at weekly or biweekly intervals during the breeding season from March through early July. Frogs, after collection, were imediately placed into a wet fishing net and were anesthetized with ether. Blood (0.8–2.0 ml) was drawn from the conus arteriosus with a 2-ml heparinized syringe, and centrifuged at 1000 G for 10 min at 8°C; and plasma (0.4–1.0 ml) was collected and stored at −20°C until androgen assay. Both right and left testes of each frog were removed and their lengths and weights were measured. One testis was fixed in Bouin's fluid for histological observations, and the other was stored at −20°C for androgen assay. Liver and fat bodies were removed and weighed as well.

Histological examination

Bouin's-fixed testes were wax-embedded, sectioned at 7 μm, and stained with Harris' hematoxylin and eosin. We determined spermatogenetic cycle by two phases: spermatogenesis, and spermiogenesis. The stages of spermatogenesis were identified according to the method used by Rastogi *et al.* [43]: stage 1, primary spermatogonia (ISPG); stage 2, secondary spermatogonia (IISPG); stage 3, primary spermatocytes (ISPC); stage 4, secondary spermatocytes (IISPC); and stage 5, spermatids (SPT). Spermiogenesis was recorded by the extent of spermatozoa present in the seminiferous tubules. Both spermatogenesis and sper-

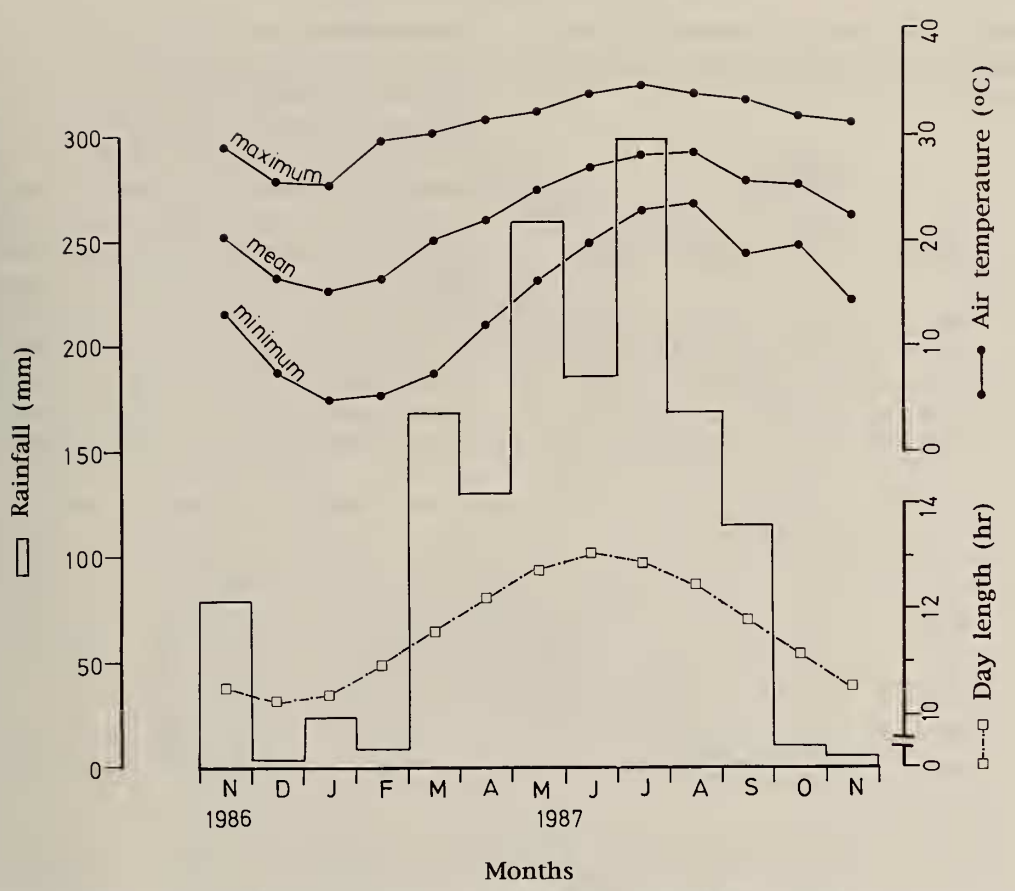


FIG. 1. Monthly cahgnes in the rainfall, photoperiod (day length), and air temperature in Tungshih, Yunlin County, Taiwan.

miogenesis of each animal were determined from twenty cross-sectioned seminiferous tubules representing four different cross-sections of each testis. The proliferation of Leydig cells in the interstitial tissues was recorded.

Radioimmunoassay of androgen in plasma and testis

Androgen concentrations in blood plasma and testis were measured by the radioimmunoassay described previously by Chen *et al.* [4]. The steroids in samples were extracted with diethyl ether. The average recoveries of the added steroids were 70.2% and 83.3% for plasma and testicular androgen, respectively, and individuals recoveries were run with each sample. The cross reactivities of the antiserum with various androgens, in relative to testosterone (100%), were: 5α-dihydrotestosterone (74%), androstenedione (1.23%), and androstenediol (0.59%) [59]. Thus, the data are expressed as "androgen" which mainly representing testosterone and dihydrotestosterone.

The coefficient of variation (CV) of the interassay was 11.0% (N=5) and that of intraassay was 6% (N=6) for plasma androgen. The CV of interassay was 9.2% (N=8) and that of intraassay was 2.4% (N=6) for testicular androgen.

Statistical analysis

Analysis of variance (ANOVA) and Duncan's multiple range tests were used to examine differences among the numerous means. Linear correlations were performed for all variables. Probability

levels of 0.05 and 0.01 were used to indicate significance in comparison of means and correlations [49].

RESULTS

Annual changes in the sizes of body, testis, liver and fat body

Changes in body weight and body length are shown in Table 1. During the annual reproductive cycle of *R. rugulosa*, body weights ranged from 30–82 g; and body lengths from 70–92 mm. The body weights were greater during May–November than those during December–April. The weight of paired testes increased during Dcember–February, peaked in March, sharply declined in April–May, and remained at low levels in June–October (Table 1). Differential changes in the weights and lengths of left and right testes were observed (Fig. 2). The mean weight of the left testis was 20% higher than that of the right testis; while, the mean length of the left testis was 40% higher than that of the right testis. The shape of left testis was less uniform than that of right testis. In addition, the annual changes in lengths of both testes were not as great as those of testicular weight although the lengths of both testes rose in February–April.

Table 1. Changes in the sizes of body, testis, liver, and fat body during an annual reproductive cycle of *R. rugulosa*

Months[1]		N[2]	Body[3]		Organ weights[3]		
			length	weight	testes	liver	fat bodies
			(cm)	(g)	(mg)	(g)	(g)
1986							
Nov	21	21	78.5±1.0	46.9±1.4	86.4±5.9	1.33±0.06	2.40±0.20
Dec	28	12	80.1±1.4	40.9±2.0	88.5±8.7	0.89±0.05	1.63±0.18
1987							
Jan	23	20	78.8±0.9	42.5±1.3	117.4±9.2	1.05±0.06	1.38±0.15
Feb	26	20	80.4±0.7	43.0±1.3	132.8±10	0.97±0.05	1.52±0.14
Mar	12	16	80.2±0.8	42.7±1.0	133.2±9.7	0.78±0.03	1.10±0.17
	26	16	82.8±0.6	47.9±1.2	131.3±4.8	0.85±0.03	1.28±0.16
Apr	2	15	79.2±0.6	38.5±0.9	115.7±8.2	0.66±0.02	0.82±0.11
	9	19	79.2±0.6	39.2±0.9	107.6±4.8	0.59±0.03	0.61±0.09
	16	14	79.4±0.7	39.4±1.2	122.1±10	0.62±0.03	0.51±0.01
	23	17	77.6±0.7	37.7±3.9	102.5±5.3	0.62±0.03	0.34±0.10
	30	16	77.5±0.7	36.2±1.0	110.6±5.2	0.58±0.03	0.29±0.06
May	7	11	78.3±1.0	39.1±1.5	109.8±6.5	0.65±0.04	0.29±0.06
	21	17	80.5±1.3	44.8±2.0	85.0±4.6	0.88±0.06	0.14±0.07
Jun	4	19	79.9±0.6	49.2±1.5	71.5±5.7	1.36±0.09	0.14±0.03
	28	21	81.3±0.7	55.1±1.9	72.6±4.2	2.07±0.14	0.19±0.03
Jul	29	25	81.3±0.8	43.8±1.2	79.4±7.1	1.28±0.07	1.07±0.22
Aug	29	22	84.8±0.7	56.6±2.5	60.8±6.3	3.14±0.28	1.35±0.17
Sep	24	17	81.6±0.9	52.8±1.2	65.0±7.4	3.40±0.28	2.96±0.25
Oct	30	12	80.6±0.6	51.1±1.9	67.3±9.4	3.00±0.27	2.42±0.14
Nov	23	17	81.3±0.7	50.7±2.0	99.0±6.4	1.68±0.11	2.39±0.26

[1] The frogs were collected monthly, or more frequently during the active breeding season from march through June.
[2] Number of animals
[3] The data are expressed as the means±SE.

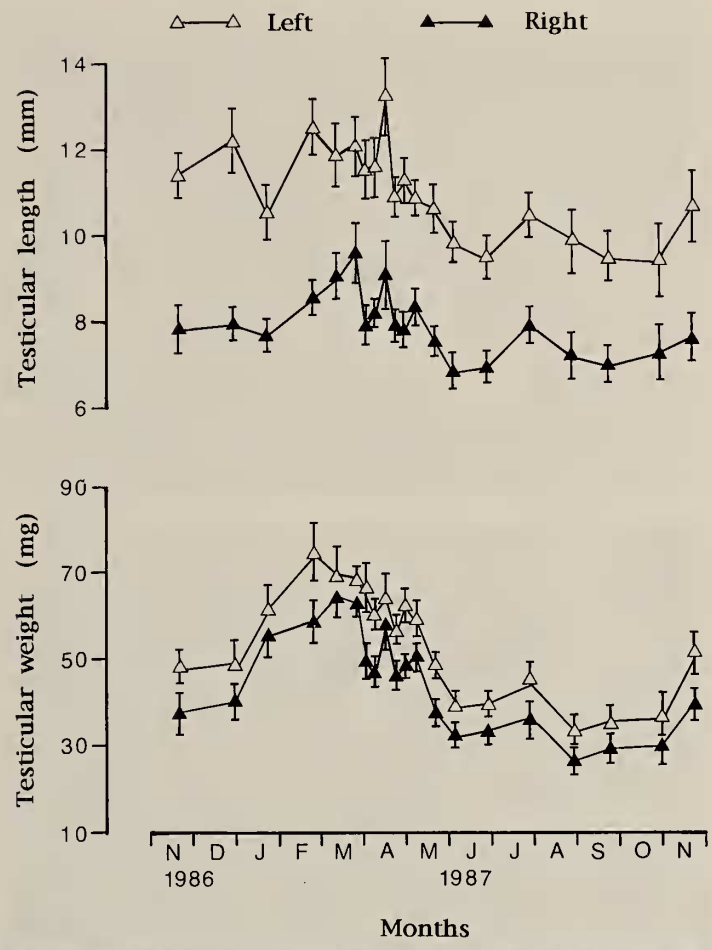


FIG. 2. Changes in the weights and lengths of the left and right testes during an annual reproductive cycle of *R. rugulosa*. The data are expressed as the means ± SE, and the number of animlas as indicated in Table 1.

Annual pattern in the weights of liver and fat body are shown in Table 1. The liver and fat body weights decreased in November–February, reached the minimal sizes in early May and late June, repsectively, and increase in early June and late July, repsectively. In September, both organs attained the maximal sizes, and then began to decrease in late October. Highly significant correlation was observed between liver and fat body weights ($r=0.60$, $p<0.01$). As shown in Fig. 3, an inverse relationship existed between the hepatosomatic index and the gonadosomatic index ($r=-0.828$, $p<0.01$). The correlation between fat body-somatic index and the gonadosomatic index was, however, not significant.

Testicular development and spermatogenesis

Proliferation of Leydig cells changed with season (Fig. 4). In November–December and January–February, Leydig cells were sparse and difficult to distinguish from other connective tissue (Fig. 4-A). The Leydig cells began to proliferate in late March and were abundant in the broad intertubule space in April–July (Fig. 4-B) after which they rapidly decreasd between the decreasing intertubular space (Fig. 4-C).

Seasonal changes in histological observations of the spermatogenetic cycle in the testis of *R. tigerina rugulosa* are also representatively shown in Fig. 4. The spermatogenetic cycle showed every

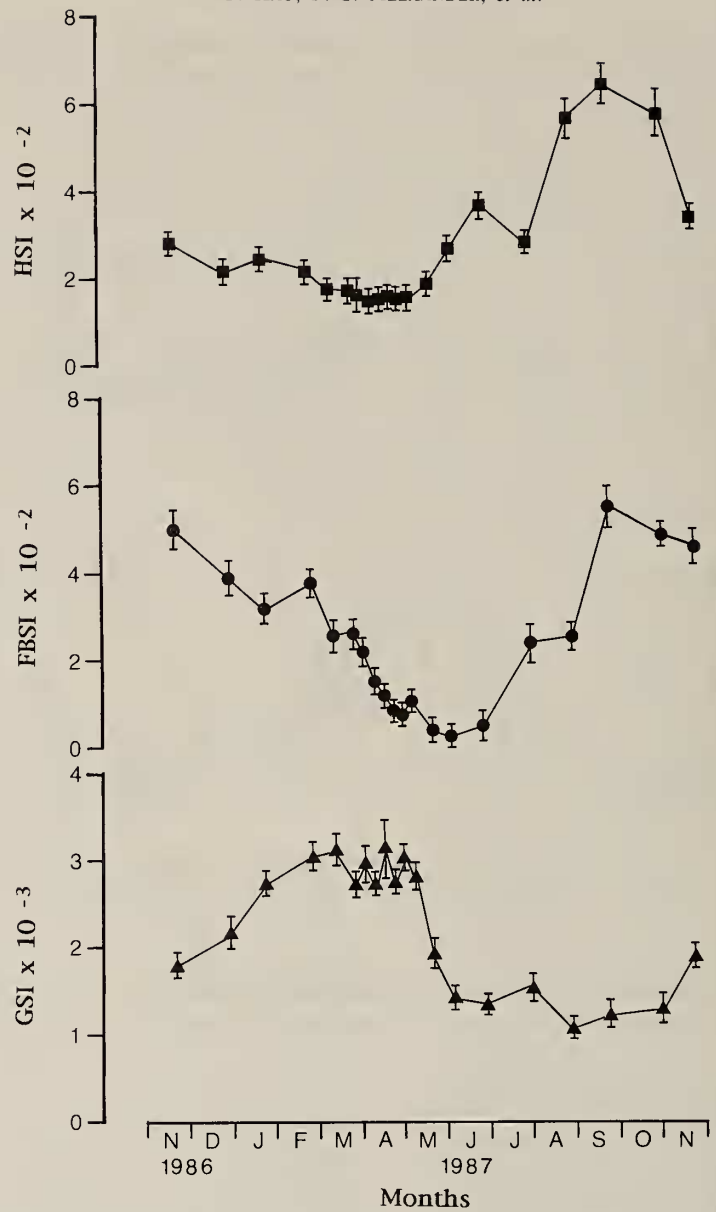
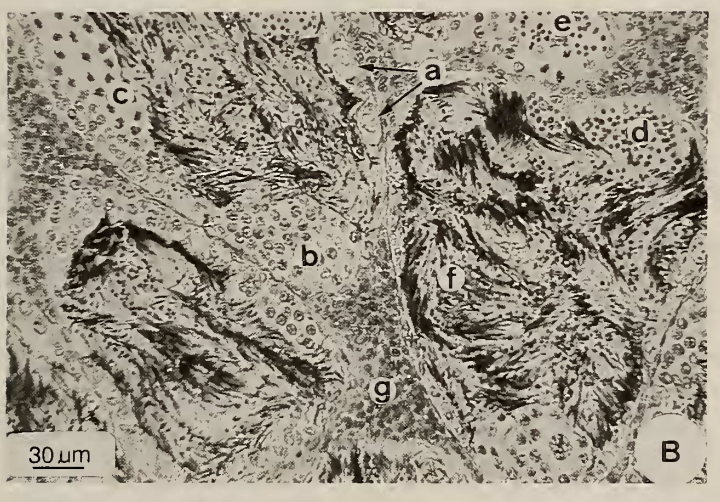
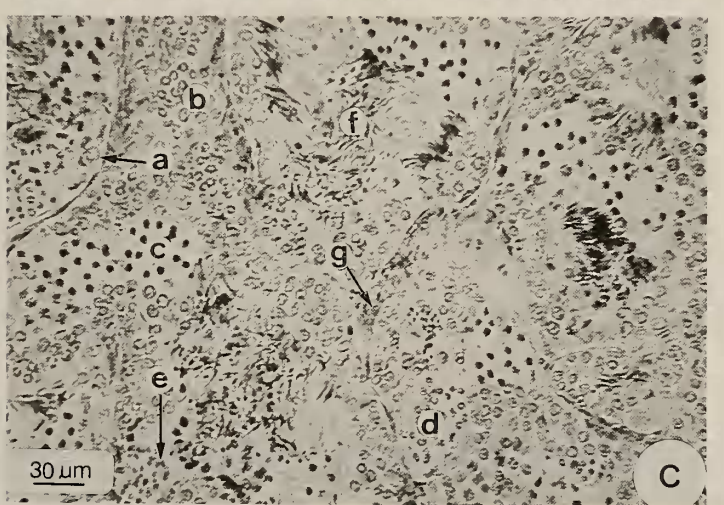


FIG. 3. The relations of liver and fat body weights to the testicular weights during an annual reproductive cycle of *R. rugulosa*. The data are expressed as mean ± SE, and the number of animals as indicated in Table 1. GSI, testes weight/body weight; HSI, liver weight/body weight; FBSI, fat body weight/body weight.

FIG. 4. Seasonal changes in the spermatogenetic cycle and the proliferation of Leydig cells of *R. rugulosa*. **a**, the nest of primary spermatogonia; **b**, the nest of secondary spermatogonia; **c**, the nest of primary spermatocytes; **d**, the nest of secondary spermatocytes; **e**, the nest of spermatids; **f**, spermatozoa; **g**, Leydig cells. A. Hibernation (December). Primary and secondary spermatogonia were the most numerous spermatogenetic cells in this period. A few clusters of spermatozoa were produced. Leydig cells existed in sparse between the seminiferous tubules. B. Breeding Season (May). Massive spermatozoa were formed in this period. Leydig cells were abundant in the intertubular space. C. Postbreeding Season (August). Cell nests of all stages of spermatogenetic cycle were existent. Few spermatozoa occurred in this period. Leydig cells were greatly reduced in number in the intertubules.


a
b
c
d
e
f
g
30 µm
A


a
b
c
d
e
f
g
30 µm
B


a
b
c
d
e
f
g
30 µm
C

stage of spermatogenesis year around. Only a few clusters of spermatozoa were found in the seminiferous tubules during hibernation (November–February) (Fig. 4-A). Massive formation of spermatozoa occurred during the active breeding season mainly in April–June (Fig. 4-B). Spermatozoa were greatly reduced in number during postbreeding in August–October (Fig. 4-C).

Significant annual changes occured in the spermatogenetic cycle (ANOVA, $p<0.01$) based on the estimate of the number of various stages of cell nests from primary spermatogonia to spermatids (Fig. 5). In November–December of 1986, ISPG were most numerous, accounting for 48% of the

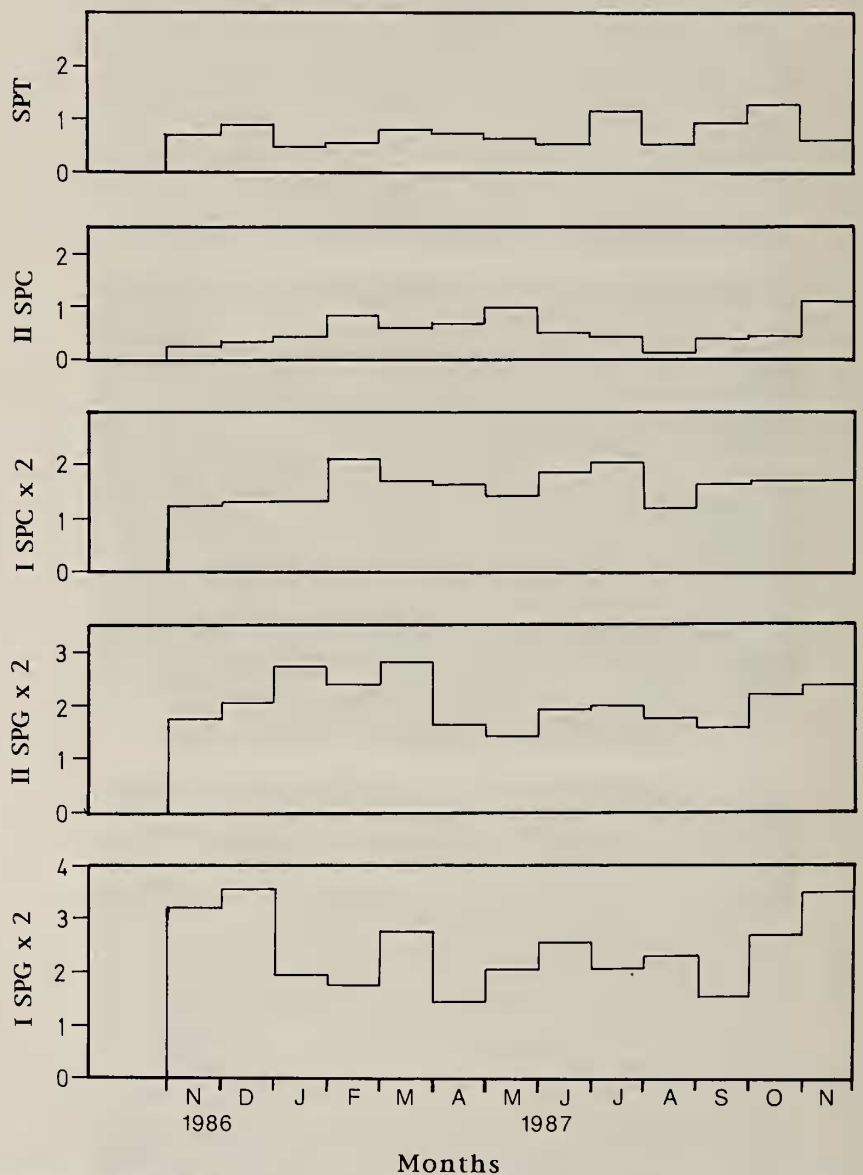


Fig. 5. Quantitative changes in different stages of spermatogenesis during an annual reproductive cycle of *R. rugulosa*. Values are expressed as the average number of cell nests of various spermatogenetic stages per seminiferous tubule. ISPG and IISPG, primary and secondary spermatogonia; ISPC and IISPC, primary and secondary spermatocytes; SPT, spermatids.

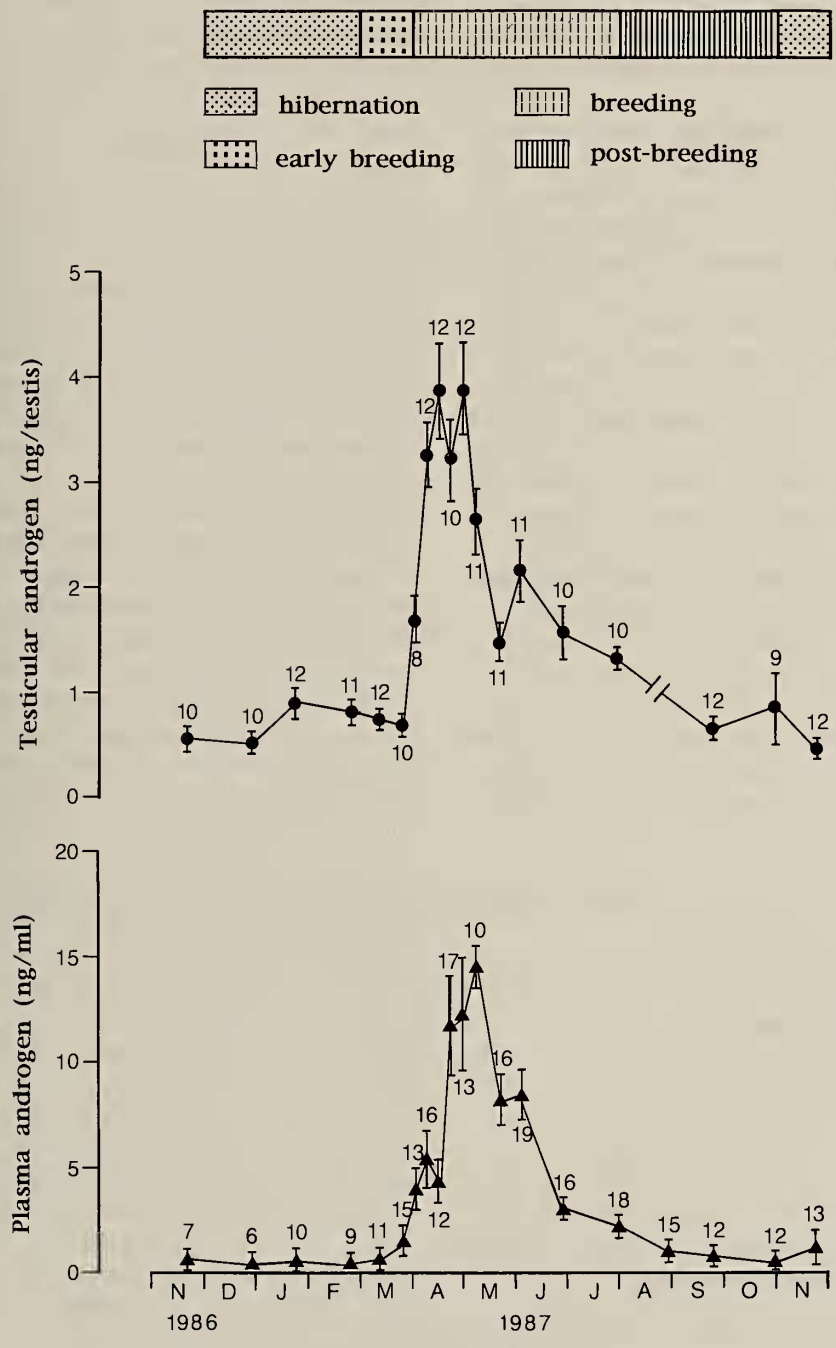


FIG. 6. The unimodal patterns of changes in testicular androgen content and plasma androgen levels during an annual reproductive cycle of *R. rugulosa*. The annual reproductive cycle was divided into four periods: hibernation, early breeding, breeding, and post-breeding. The data are expressed as the means ± SE, and the number of animlas as indicated with Arabic numerals.

total germinal cell nests; other stages of germ cells began to multiply in December. In January and February, the number of ISPG and SPT declined, but those of IISPG, ISPC, and IISPC increased with the rise in ISPC and IISPC lagging behind that of IISPG. In March there were increases in the ISPG and IISPG followed by sharp decreases in April. ISPG then exhibited a slight increase with some fluctuations in May–August; it decreased again in September, followed by sharp increases in October and November. IISPG remained virtually constant from May through September, and then increased in October. ISPC decreased in March–May, and increased again in June–July; by contrast, a reverse pattern existed for IISPC in March–July. At this period (March–July), the cell nests of SPC (the sum of ISPC and IISPC) became the most numerous. During the breeding season (March–July), spermatozoa were massively formed from SPT. ISPC, IISPC, SPT, and spermatozoa all reduced in August, and increased in September–October.

Plasma and testicular androgen levels

Plasma and testicular androgen levels showed significant seasonal changes (ANOVA, $p<0.05$, Fig. 6). Plasma androgen levels were very low (<1 ng/ml) during hibernation (November–February), increased in late March, peaked in early May (14.50 ± 1.05 ng/ml), decreased sharply in late May and June, and reached basal levels afterwards (from July through October). Testicular androgen content was also low during hibernation period (<1 ng/testis), increased rapidly in early April (3.28 ± 0.28 ng/testis) and peaked in middle (3.88 ± 0.45 ng/testis) and late April (3.90 ± 0.44 ng/testis); the androgen levels were decreased markedly in May, followed by a gradual drop afterwards, and reached basal values in September (0.7 ± 0.07 ng/testis). During the annual reproductive cycle of *R. rugulosa,* the difference between maximal and minimal values in testicular androgen content was 10 fold, while that in plasma androgen was 50 fold.

Correlations of plasma androgen concentration with testicular development, liver, and fat body are summarized in Table 2. Highly significant correlations were observed between plasma androgen levels and testicular androgen content. There were significant negative correlations of plasma and testicular androgen levels with liver weight and fat body weight respectively, as well as with two stages of spermatogonia (the number of nests of primary spermatogonia, ISPG, and of

Table 2. Correlation coefficients (r) of plasma and testicular androgen levels with spermatogenetic cell types, and liver and fat body weights of male *R. rugulosa* during an annual reproductive cycle

	Plasma androgen	Testicular androgen
Testicular androgen	0.734**	1.0
Testicular weight	0.087	0.188
GSI	0.288	0.426
Liver weight	−0.438†	−0.460*
HSI	−0.448†	−0.466†
Fat body weight	−0.702**	−0.696**
FBSI	−0.719**	−0.707**
Primary spermatogonia	−0.515†	−0.628*
Secondary spermatogonia	−0.499†	−0.403
Primary spermatocytes	0.025	0.139
Secondary spermatocytes	0.389	0.141
Spermatids	−0.074	−0.019

Correlation coefficients (r) were analyzed from the monthly means of those parameters. GSI, gonadosomatic index=testis weight/body weight; FBSI, fat body weight/body weight; HSI, liver weight/body weight; †, $p<0.10$; *, $p<0.05$; **, $p<0.01$.

secondary spermatogonia, IISPG). The rising phases of both plasma and testicular androgen levels were synchronized with active spermiogenesis (Figs. 4-B and 6).

DISCUSSION

Annual patterns of testis, liver, and fat body

A unimodal pattern of annual variation exists in the testicular weight of *R. rugulosa.* There are significant differences between the maximal and minimal mean values of both testicular weight (2 fold) and GSI (3 fold) during the annual cycle. Such annual changes in GSI of this species are similar to those of *R. tigerina* [44], *B. marinus* [44], *R. esculenta* [21, 43], and *B. japonicus* [13, 30], but are different from those of *R. perezi* [6] and *R. catesbeiana* [19, 58], showing little variation of GSI. The annual change in the testicular weight of *R. rugulosa* also shows a positive correlation with secondary spermatogonia ($r=0.625$; $p<0.05$). Thus, the rising phase of testicular weight in January–March is presumably due to the increases in the secondary spermatogonia even though other factors, such as water reabsorption and cellular hypertrophy, may be involved. On the other hand, the observations that the testicular weight of *R. rugulosa* exhibited some fluctuations during the entire breeding season (March–July) and sharp decreases in the late breeding season (June–early July) may be attributed to the evacuation of spermatozoa. We observed that both lengths and weights of left testis were greater than those of right testis during an annual cycle of *R. rugulosa.* Such findings are similar to those of *B. bufo* and *R. nigromaculata* reported by other investigators [53].

In male anurans, the roles of liver in the reproductive cycle include the formation of sex steroid-binding proteins, the formation and storage of lipids and carbohydrates for the metabolic activity of the gonads, the degradation of gonadal steroid hormones and others [23, 26, 29, 35, 47]. we demonstrated that a unimodal pattern of annual change in liver weight exists in male *R. rugulosa.* This finding is similar to those of *A. crepitans* [23], *B. woodhousei* [23], *B. canorus* [29], and *R. esculenta* [47], but is different from that of *R. nigromaculata* [27], a temperate species, which showing no significant difference in the liver weight during the annual cycle. We also found that the annual change in liver weight of *R. rugulosa* positively correlates with that of the body weight ($r=0.877$, $p<0.01$), but negatively with that of the testicular weight. Such findings are in disagreement with those reported for *R. ridibunda* [24], which displaying high positive correlations among the weights of body, liver, and testes.

The functions of fat body on reproduction in male anurans are likely the supply of metabolic energy for the maintenance of testicular activity during hibernation, and for androgen production during the breeding season, as demonstrated in *R. esculenta* [28, 43], and in *Acris crepitans* and *B. woodhousei* [23]. Male *R. rugulosa,* as observed in the present study, exhibits a unimodal pattern of annual change in the fat body weight, which being positively correlated with liver weight, but not with the body weight ($r=0.421$, $p>0.05$). The annual change in the fat body weight is not correlated with that in the testicular weight, but is negatively correlated with androgen levels in testis and plasma (Table 2). These results are similar to those of *R. perezi* [6] and *B. japonicus* [30] thus support the proposals that the fat bodies serve as a major lipid nutrient for metabolic energy in general and in the testis in particular [5, 8].

Spermatogenetic cycle

The annual spermatogenetic cycles in anurans have been classified into three types: discontinuous, potentially continuous, and continuous [22]. Discontinuous spermatogenesis is commonly found in temperate and cold zone species such as *R. temporaria* [22]; while continuous spermatogenesis is characteristic of the spermatogenetic cycle of tropical and subtropical anurans [22]. Some anuran species such as *R. esculenta* and *R. tigerina* are classified as "potentially continuous cycle" where the primary spermatogonia never become refractory to gonadotropin and continue to undergo multiplication until the lowering temperature of autumn and winter causes a retardation [1, 22, 34, 44, 45]. In the present study, we observed that *R. rugulosa* inhabiting subtropical Taiwan exhibits a continuous spermatogenetic

cycle with germ cells of all stages found throughout the year, although massive spermatozoa being existed in April–May.

Androgen patterns

Annual changes in plasma androgen levels have been studied in many species of male anurans: seven temperate species, *R. perezi* [6], *R. catesbeiana* [19], *B. japonicus* [13], *R. esculenta* [7], *B. mauritanicus* [48], *R. ridibunda* [55], and *R. nigromaculata* [51]; three subtropical species, *P. dacnicolor* [41] and *B. bufo gargarizans* and *B. melanostictus* [12]; a tropical species, *D. occipitalis* [17]. These male anurans exhibit either unimodal or bimodal types of annual patterns in plasma androgen characterized by a single peak or two peaks, respectively, of annual circulating androgen levels [13]. Studies have demonstrated that subtropical male anurans show an annual unimodal pattern of plasma androgen levels [12, 41], while temperate male anurans show both unimodal and bimodal patterns of androgen levels [6, 7, 13, 19, 48, 51, 55]. However, *D. occipitalis* [17], a tropical anura, displays a bimodal pattern of annual plasma androgen level. We have demonstrated in the present study that male *R. rugulosa,* inhabiting subtropical Taiwan, displays a unimodal pattern of circulating androgen levels during an annual reproductive cycle. This pattern is comparable with those of subtropical anurans reported by other investigators [12, 41].

In vitro studies of testicular androgen biosynthesis in Amphibia indicate that dihydrotestosterone (DHT) is the major androgen in Anura and that testosterone (T) is the major androgen in Urodele [15]. Such a conclusion is supported by *in vivo* investigations of the circulating androgens in Anurans, *R. catesbeiana* [19], *R. nigromaculata* [51], *R. pipiens* [57], *Eleutherodactylus coqui* [54], and in Urodeles, *Necturus maculosus* [2], *Salamandra salamandra* [18], *Pleurodeles waltl* [10], and *Ambystoma tigrinum* [33]. However, in several anuran species, such as *B. japonicus* [13], *R. esculenta* [38], *B. mauritanicus* [48], and *P. dacnicolor* [41], T is more predominant than DHT in circulation. Whether T or DHT is the predominant androgen in the male Chinese bullfrog, *R. rugulosa,* requires further investigations. In mammals, DTH is identified to be primarily a metabolite of T in peripheral target tissues. DTH is considered to be directly formed by testis of anuran amphibians [11, 15, 16, 31, 32, 36, 37], while it has not been identified in the testis of urodel amphibians [15, 25, 29, 50, 52]. The physiological role of DHT in amphibians is not yet clearly defined, and thus needs further studies.

Androgen secretion is regulated by the interactions of hormonal and environmental factors [43, 44]. Rastogi *et al.* [43] reported that androgen in *R. esculenta* shows a negative feedback interaction with gonadotropins by a study of hypophysectomic implantation with homoplastic pituitary extract; on the other hand, in *R. catesbeiana,* plasma gonadotropins and steroids are simultaneously elevated throughout most of the reproductive cycle, as well as LH levels exhibit a weak correlation with androgen levels [19]. In *B. japonicus,* Itoh *et al.* [13] reported that plasma LH levels, but not FSH levels, were highly correlated with circulating androgen levels.

Correlation of androgen patterns with spermatogenetic cycle

The hormonal control of spermatogenesis in anurans has been investigated [6, 40, 41, 43, 44, 58]. It was demonstrated that FSH stimulates the spermatogenesis on the basis of the studies with mammalian hormones [40, 44]. On the other hand, the controversial results of the effect of androgen on spermatogenesis have been reported in anurans: such as in *R. hexadactyla, R. esculenta* and *R. tigerina,* where androgen exhibiting suppression of spermatogenesis (mainly at the secondary spermatogonia stage); and in *B. fowleri* and *B. arenarum,* where androgen showing the stimulation of spermatogenesis [1, 40, 44]. In male *R. rugulosa,* as observed in this study, androgen levels in both testis and plasma show a weakly reverse correlation with spermatogenetic stages of primary and secondary spermatogonia, but parallel with changes in the number of spermatozoa. The role which androgen plays on different stages of spermatogenesis in *R. rugulosa* needs further investigation.

Relations of androgen patterns to breeding activity

In male *R. rugulosa,* we observed that the pattern of androgen secretion parallels with breeding activity; the circulating androgen peak occurring in early May was found to be coincident with the active breeding period in April–June. Such patterns are similar to those of *P. dacnicolor* [41], but different from those of *R. perezi* [6], *B. japonicus* [13], *R. esculenta* [38], and *R. ridibunda* [55] which showing a peak of plasma androgen level prior to breeding season. The patterns of testicular activity and androgen secretion observed in male *R. rugulosa* were synchronized with the patterns of ovarian maturation and estradiol secretion of the females, which were simultaneously investigated in a parallel study at the same frog culture farm [3].

The role of androgen in induction and subsequent maintenance of male reproductive behavior, has been established in several anurans [14, 48, 54, 57]. For example, *B. Mauitanicus* [48] and *E. coqui* [54] both show a high androgen level during the amplexing behavior; while, *B. japonicus* [14] shows a high androgen level during the migration behavior. We also observed in the present study that high androgen levels in plasma of *R. rugulosa* are paralleled with its reproductive activity such as chorussing and spermiation, as well as with nuptial pad development in relation to amplexing behavior. These observations support the proposal that androgen plays a role in maintenance of the male reproductive behavior in this species.

Association of environmental factors with reproductive cycle

Effects of environmental cues on male anuran reproductive cycle have been studied [6, 22, 42, 43, 44, 46]. It was reported that, in most subtropical and tropical species, rainfall is the most important factor associated with initiation of breeding activity since temperature changes little with seasons in these areas [11]. However, the mechanisms of rainfall in the regulation of anuran reproductive cycle are not clear [46]. On the other hand, temperature is the primary factor in temperate species, which exhibiting a cyclic reproductive pattern with season. The role of temperature in the regulation of anuran spermatogenetic cycle has been investigated in many speices [6, 22, 43, 46]. In *R. esculenta,* the proliferation of primary spermatogonia is available in low temperature; in addition, the formation of secondary spermatogonia, and primary and secondary spermatocytes occurs with increasing temperature [42, 43]. It was subsequently demonstrated in this species that the temperature plays a major role in the spermatogenesis via stimulation of the release of pituitary gonadotropins and testicular androgen secretion [43]. Rastogi *et al.* [43] also demonstrated that photoperiod serves as a permissive role on temperature influence upon spermatogenesis of *R. esculenta.* In the present study, we found that the rising phases of climatic factors (rainfall, temperature, and photoperiod) parallel temporally with active testicular development and spermatogenesis, the rising androgen levels in plasma and testis, and breeding activity during the annual reproductive cycle of *R. rugulosa.* Regulatory mechanisms of environmental factors on a reproductive cycle of anurans are highly complex, and are presumably mediated by a pathway of optic or other transmissions to the hypothalamo-hypophysial-gonadal axis.

ACKNOWLEDGMENTS

We are grateful to the Department of Biology, Tunghai University, Taichung, and Institute of Zoology, Academia Sinica, Taipei, Taiwan, R. O. C., for the grants supporting this study. Thanks are also due to Mr. Shen San-tai, Miss Chen Chao-yin, Miss Chern Jyun-rur, and Miss Su Huey-min for assistance during various periods of this study.

REFERENCES

1 Basu SL (1962) A study of the spermatogenesis of common Indian frog (*Rana tigerina*) treated with testosterone. Naturwissenschaften 49: 188

2 Bolaffi JL and Callard IP (1979) Plasma steroid profiles in male and female mudpuppies, *Necturus maculosus* (Rafinesque). Gen Comp Endocrinol 37: 443–450

3 Chen CY (1988) Seasonal changes in ovarian activity and plasma sex steroids levels in the Chinese bullfrog, *Rana tigerina rugulosa* Wiegmann. Master's Thesis, Tunghai University, Taichung, Taiwan, ROC

4 Chen RH, Lin JY, Yu YL and Cheng HY (1987) Annual changes in plasma and testicular androgen in relation to reproductive cycle in a Japalura Lizard in Taiwan. Zool Sci 4: 323–329

5 Chieffi G, Rastogi RK, Iela L and Milone M (1975) The function of fat bodies in relation to the hypothalamohypophyseal-gonadal axis in the frog, *Rana esculenta.* Cell Tiss Res 161: 157–165

6 Delgado MJ, Gutierrez P and Alonso-Bedate M (1989) Seasonal cycle in testicular activity in the frog, *Rana perezi.* Gen Comp Endocrinol 73: 1–11

7 D'Istria M, Delrio G, Botte V and Chieffi G (1974) Radioimmunoassay of testosterone, 17β-oestradiol and oestrone in the male and female plasma of *Rana esculenta* during sexual cycle. Steroids Lipids Res 5: 42–48

8 Fitzpatrick LC (1976) Life history patterns of storage and utilization of lipids for energy in amphibians Amer Zool 16: 725–732

9 Frost DR (1985) Amphibian Species of the World. Lawrence, Kansas: Assoc Syst Coll pp 512–518

10 Garnier DH (1985) Androgen and estrogen levels in the palsma of *Pleurodeles waltl,* Michah, during the annual cycle. I. Male cycle. Gen Comp Endocrinol 58: 376–385

11 Gavaud J (1975) Etude experimentale du role des facteur externes sur la spermatogenese et la steroidogenese des male de *Nectophrynoides occidentalis* Angel J Physiol (Paris) 70: 549–559

12 Huang, WS (1991) Male reproductive cycles of two sympatric toads, *Bufo bufo gargarizas* and *Bufo melanostictus.* Master's Thesis, Tunghai University, Taichung, Taiwan, ROC

13 Itoh M, Inoue M and Ishii S (1990) Annual cycle of pituitary and plasma gonadotropins and plasma sex steroids in a wild population of the toad, *Bufo japonicus.* Gen Comp Endocrinol 78: 242–253

14 Itoh M and Ishii S (1990) Changes in plasma levels of gonadotropins and sex steroids in the toad, *Bufo japonicus,* in association with behavior during the breeding season. Gen Comp Endcrinol 80: 451–464

15 Kime DE (1980) Comparative aspects of testicular androgen biosynthesis in nonmammalian vertebrates. In "Steroids and Their Mechanism of Action in Nonmammalian Vertebrates". Eds by G Delrio and J Brachet, Raven Press, New York pp 17–32

16 Kime DE and Hews EA (1978) Androgen biosynthesis *in vitro* by testes from amphibia. Gen Comp Endocrinol 35: 280–288

17 Kuhn ER, Gevaerts H, Vandorpe G and Jacobs GFM (1987) Plasma concentrations of testosterone and thyroxine in males of the giant swamp frog, *Dicroglossus occipitalis* at the equator. Gen Com Endocrinol 68: 492–493

18 Lecouteux A, Garnier DH, Bassez T and Joly J (1985) Seasonal variations of androgens, estrogens, and progestorone in the different lobules of the tesits and in the plasma of *Salamandra salamandra.* Gen Comp Endcrinol 58: 211–221

19 Licht P, McCreery BR, Barnes R and Pang R (1983) Seasonal and stress related changes in plasma gonadotropins, sex steroids, and corticosterone in the bullfrog, *Rana catesbeiana.* Gen Comp Endocrinol 50: 124–145

20 Liu CC (1950) Amphibians of Western China. Fieldiana: Zoology Memoirs 2: 1–400

21 Lofts B. (1964) Seasonal changes in the functional activity of the interstitital and spermatogenetic tissues of the green frog, *Rana esculenta.* Gen Comp Endocrinol 4: 550–562

22 Lofts B (1974) Reproduction. In "Physiology of the Amphibia". Ed by B Lofts, Academic Press, New York Vol 2 pp 107–218

23 Long DR (1987) A comparison of energy substrates and reproductive patterns of two anurans, *Acris crepitans* and *Bufo woodhousei.* Comp Biochem Physiol 87A (1): 81–91

24 Loumbourdis NS and Kyriakopoulou-Sklavounous P (1991) Reproductive and lipid cycles in the male frog *Rana ridibunda* in northern Greece. Comp Biochem Physiol 99A: 577–583

25 Lupo di Prisco C, Basile C, Delrio G and Chieffi G (1972) *In vitro* metabolism of cholesterol-4-C^{14} and testosterone-4-C^{14} in testes and fat bodies of *Triturus cristatus carnifex.* Comp Biochem Physiol 41B: 245–249

26 Martin B (1980) Steroid-protein interactions in nonmammalian vertebrates: distribution, origin, regulation, and physiological significance of plasma steroid binding proteins. In "Steroids and Their Mechanisms of Action in nonmammalian Vertebrates". Eds by G Delrio and J Brachet, Raven Press New York pp 63–73

27 Maruyama K (1979) Seasonal cycles in organ weights and lipid levels of the frog, *Rana nigromaculata.* Annot Zool Japon 52(1): 18–27

28 Milone M, Chieffi G, Caliendo MF and Pierantoni R (1984) Effect of the removal fat bodies on lipid biosynthesis in frog testis. Boll Zool 51: 75 (Abstract)

29 Morton MC (1981) Seasonal changes in total body lipid and liver weight in the Yosemite toad. Copeia 1981(1): 234–238

30 Moriguchi Y and Iwasawa H (1987) Annual changes in male reproductive organs in *Bufo japonicus formosus*: Histological observation. General Endocrinol 6: 115–120

31 Muller CH (1975) *In vitro* steroidogenesis by bullfrog testis. Soc Study Reprod 8th Ann Meeting pp 95–96 (abstract 104)

32 Muller CH (1976) Secretion of 5α-dihydrotestosterone by bullfrog testis. Prog Abstr 58th Ann Meet-

ing Endocrine Soc p 286 (abstract 423)

33 Norris DO, Norman MF, Pancak MK and Duvall D (1985) Seasonal variations in spermatogenesis, testicular weights, vasa deferentia, and androgen levels in neotenic male tiger salamanders, *Ambystoma tigrinum*. Gen Comp Endocrinol 60: 51–57

34 Oordt PGWJ van (1960) The influence of internal and external factors in the regulation of the spermatogenetic cycle in amphibia. Symp Zool Soc London 2: 29–52

35 Ozon R (1972) Androgens in fishes, amphibians, reptiles, and birds. In "Steroids in Nonmammalian Vertebrates". Ed by DR Idler, Academic Press, New York pp 329–389

36 Ozon R, Breuer H and Hisboa BP (1964) Etude du metabolisme des hormones steroides chez les vertebre inferieurs. III Metabolisme *in ivtro* de la testosterone par l'ovaire et la testicule de la grenouille *Rana temporaria*. Gen Comp Endocrinol 4: 577–583

37 Ozon R and Stocker C (1974) Formation *in vitro* de 5α-dihydrotestosterone par le testicular de *Discoglossue pictus*. Gen Comp Endocrinol 23: 224–236

38 Pierantoni R, Iela L d'Istria M, Fasano S, Rastogi RK and Delrio G (1984) Seasonal testosterone profile and testicular responsiveness to pituitary factors and gonadotrophin-releasing hormone during two different phases of the sexual cycle of the frog (*Rana esculenta*) J Endocrinol 102: 387–392

39 Pope CH and Boring AM (1940) A survey of Chinese Amphibia. Peking Nat Hist Bull 15: 49–50

40 Rastogi RK and Iela L (1980) Steroidogenesis and spermatogenesis in anuran amphibia: A brief survey. In "Steroids and Their Mechanism of Action in Nonmammalian Vertebrates". Eds by G Delrio and J Brachet, Raven Press, New York pp 131–146

41 Rastogi RK, Iela, L, Delrio G and Bagnara JT (1986) Reproduction in Mexican leaf frog, *Pachymedusa dacnicolor* II. male Gen Comp Endocrinol 62: 23–35

42 Rastogi RK, Iela L, Delrio G, Dimeglio M, Rosso A and Chieffi G (1978) Environmental influence on testicuiar activity in the green frog, *Rana esculenta*. J Exp Zool 206: 49–64

43 Rastogi RK, Iela L, Saxena PK and Chieffi G (1976) The control of spermatogenesis in the green frog, *Rana esculenta*. J Exp Zool 196: 151–166

44 Saidapur SK (1983) Patterns of testicular activity in Indian amphibians. Indian Rev Life Sci 3: 157–184

45 Saidapur SK and Nadkarni VB (1975) Seasonal variation in the structure and function of testis and thumb pad in the frog *Rana tigerina* (Daud.). Indian J Exp Biol 13: 432–438

46 Salthe SN and Mecham JS (1974) Reproductive and courtship patterns. In "Physiology of the Amphibia". Ed by B Lofts, Academic Press, New York Vol 2 pp 309–521

47 Schlaghecke R and Blum V (1978) Seasonal variations in liver metabolism of the green frog *Rana esculenta* (L). Experientia 34: 456–457

48 Siboulet R (1981) Variations saisonnieres de la teneur plasmatique en testosterone et dihydrotestosterone chez le crapaud de mauritanie (*Bufo mauritanicus*). Gen Comp Endcrinol 43: 71–75

49 Steel RGD and Torrie JH (1980) In "Principles and Procedures of Statistics", 2nd ed McGraw-Hill Book Company

50 Tajima H, Arai R, Tomaoki B-I and Hanaoka K-I (1969) *In vitro* steroidogenesis in testicular homogenates of the Japanese newt, *Cynops Pyrrhogaster* (Boie). Gen Comp Endcrinol 12: 549–555

51 Tanaka S, Iwasawa H and Wakabayashi K (1988) Plasma levels of androgens in growing frogs of *Rana nigromaculata*. Zool Sci 5: 1007–1012

52 Tanaka S and Takikawa H (1983) Seasonal changes in plasma testosterone and 5α-dihydrotestosterone levels in adult male newt, *Cynops pyrrhogaster pyrrhogaster*. Endocrinol Japon 30(1): 1–6

53 Ting HP and Boring AM (1939) The seasonal cycle in the reproductive organs of the Chinese toad *Bufo bufo* and the pond frog *Rana nigromaculata*. Peking Nat Hist Bull 14: 49–80

54 Townsend DS and Moger WH (1987) Plasma androgen levels during parental care in a tropical frog (*Eleutherodactylus*). Horm Behav 21: 93–99

55 Vandorpe G, Jacobs GFM and Kuhn ER (1987) Seasonal changes of the 5′-monodeiodination activity in kidney and skin homogenates of male *Rana ridibunda*: relation to plasma thyroxine (T4) and testosterone. Gen Comp Endocrinol 68: 163–169

56 Varriale B, Pierantoni R, Matteo LD, Minsucci S, Fasano S, D'Antonio M and Chieffi G (1986) Plasma and testicular estradiol and plasma androgen profile in the male frog *Rana esculenta* during the annual cycle. Gen Comp Endocrinol 64: 401–404

57 Wada M, Wingfield JC and Gorbman A (1976) Correlation between blood level of androgens and sexual behavior in male leopard frogs, *Rana pipiens*. Gen Comp Endocrinol 29: 72–77

58 Yoneyama H and Iwasawa H (1985) Annual changes in the testis and accessory sex organs of the bullfrog, *Rana catesbeiana*. Zool Sci 2: 229–237

59 Yu JYL, Liaw JJ, Chang CL, Lee SN and Chen MC (1988) Seasonal changes in circulating levels of gonadal steroids during an estrous cycle of holstein-friesian cows Bull Inst Zool Academia Sinica (ROC) 27(2): 133–143